Ecotoxicological effects of lithium chloride on *Lyngbya wollei*

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Braxton Grey Crews

BS, Georgia Southwestern State University, 2018

This thesis, "Ecotoxicological Effects of Lithium Chloride on *Lyngbya wollei*," by Braxton Crews, is approved by:

Thesis Committee Chair

Dr. James A. Nienow, Ph.D.

Professor of Biology

Committee Member

Dr. Eric Chambers, Ph.D.

Associate Professor of Biology

Committee Member

Dr. Thomas Manning, Ph.D.

Professor of Chemistry

Associate Provost for Graduate Studies and Research

Becky K. da Cruz, Ph.D., J.D.

Professor of Criminal Justice

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Abstract

Lyngbya wollei is a filamentous cyanobacterium that plagues freshwater environments of the Southeastern United States because it impedes freshwater navigation and recreation, and produces harmful cyanotoxins along with a foul odor. Current treatment plans for *L. wollei* blooms include numerous biological and mechanical methods, while chemical control is primarily limited to application of copper- and aluminum-based compounds that often have significant negative side effects. Here, we investigate the potential of lithium compounds as control agents for *L. wollei*. Lithium is not expected to bioaccumulate, has terrific bioremediation potential, and is relatively non-toxic, exhibiting qualities that make it an excellent candidate for an agent of chemical algal control. In this experiment, cultures of *L. wollei* were exposed to lithium chloride in concentrations increasing up to 400 mg/L. Additionally, filamentous freshwater green algae were also treated with lithium chloride, in order to gain some insight into the specificity of lithium chloride for *L. wollei*. In a separate set of experiments, *L. wollei* filaments were exposed to the antibiotics streptomycin, tetracycline, and isoniazid, and to equivalent concentrations of potassium chloride. Filaments were checked for damage using a light microscope, and PAM-fluorometry was used to investigate changes in photosynthetic activity. The effect concentration of lithium chloride was determined to be between 100-200 mg/L for *L. wollei* and >400 mg/L for freshwater green algae. The antibiotics did not effectively damage the filaments, but the potassium chloride solution produced damage at the effect concentration of lithium, indicating a promising field for further investigation. PAM-fluorometry results suggest that the mechanism by which lithium chloride damages and kills cells in the filaments is unrelated to photosynthesis- the effects observed could be attributed to seasonal variation in *L. wollei*. In the highest concentration treatment group, freshwater microorganisms

were observed, suggesting that lithium chloride may be a safe and effective means of chemical control of freshwater cyanobacteria, pending further experimentation.

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Introduction

Some species of freshwater cyanobacteria are known to produce both hepatotoxic and neurotoxic cyanotoxins that have negative impacts on the health of humans and other mammals (Kaur 2019). Some freshwater cyanotoxin-producing cyanobacteria such as *Microcystis* spp. exist as single cells or colonies (Harke et al. 2016) while others are filamentous (Komarek and Johansen 2015). One such species of filamentous cyanobacteria, *Lyngbya wollei* (which belongs to family Oscillatoriaceae), is easily identifiable by its large size (up to 50 μm in diameter), thick polysaccharide sheath, and thin, discoid cells (Speziale and Dyck 1992). *L. wollei* is widespread in the United States, and has been found to produce cylindrospermopsin and its analogue deoxy-cylindrodpermopsin (Seifert et al. 2007). Additionally, it causes paralytic shellfish poisoning through the production of saxitoxins such as decarbamoyl 2 and 3 (Carmichael et al. 1997). These compounds work by blocking sodium channels on motor nerves (Gad 2014) and can lead to flaccid paralysis (Dettbarn 1971).

Lyngbya wollei has a distribution that ranges from the Southern U.S. to Canada (Bridgeman and Penamon 2010), making it relatively easy to find, collect, and study. Particularly, it is a problem in the Southeastern United States because it exhibits optimal growth in an environment with a slightly alkaline pH (maximum growth at pH 8.0), a temperature range between 7-20°C (Panek 2012), and optimal biomass and cyanotoxin production at a temperature of approximately 26°C (Hudon et al. 2014), and very low salinity (Cowell and Botts 1994), conditions characteristic of bodies of freshwater in the Southeastern United States throughout much of the year. Its proliferation is favored by organic compounds in the water (Levesque et al. 2012). Areas colonized by *L. wollei* are

anticipated to increase under various climate change scenarios (Levesque et al. 2015). *L. wollei* often persists in benthic freshwater environments year-round, due to abundant phycobilin pigments adapted for interception of a broad spectrum of light at low intensities (Speziale et al. 1991). Blooms of the species occur in warmer climates (Paerl and Huisman 2008), which can impede freshwater navigation and recreation, clog water intakes, and produce volatile organic compounds that cause a foul odor and alter drinking water taste (Vulgamore et al. 2019). *Lyngbya wollei* forms large floating mats in freshwater ponds and lakes during the summer and benthic aggregations in cooler temperatures, and it has morphological defenses to reduce predation (Camacho and Thacker 2006). Aquatic ecosystems composed primarily of *L. wollei* are often characterized by a lower biomass of invertebrates and large fish, lower fish species richness, and slower-growing juvenile fish when compared to aquatic ecosystems composed primarily of macrophyte species (Hudon et al. 2014). However, it has been found to have less biomass and toxicity in environments with low nitrogen and phosphorous concentrations (Yin et al. 1997).

Non-chemical algae treatments are commonly used in smaller bodies of freshwater, but they are not very effective. Physical removal of algae can eradicate most of the populations in ponds, but this process is very labor intensive. Biological removal is a strategy that is sometimes used as well, primarily via the grass carp. This method is quite ineffective, however, as grass carp prefer aquatic plants over algae, and there are concerns over their potential to invade ecologically sensitive areas (Chilton II and Muoneke 1992). The introduction of these fish can also have the opposite effect than is intended, as macrophyte removal can lead to increases in green algal and cyanobacterial

biomass (Maceina et al. 1992). One additional (and by far least effective) strategy of benthic algae growth control includes depending on natural phenomena to inhibit benthic algal growth, and this is limited to flowing bodies of water. It is suggested that mild flood events (that do not remove organisms from substrates) inhibit algal growth in nutrient-poor streams, but stimulate algal growth in nutrient-rich streams (Humphrey and Stevenson 1992).

The most common method used to control the growth of benthic algae such as *L. wollei* in lakes, ponds, and pools is treatment with chemical algaecides. Many of these algaecides are copper-based, but there are also many that are used that are sodium-based (primarily used by pool owners that want to protect their pool surfaces from oxidation that is sped up when copper is added). Copper-based algaecides contain the active ingredients copper sulfate and/or chelated copper. Copper sulfate is an effective algaecide, but it can change the biogeochemical structure of lakes and ponds and lead to a loss of seasonal variability (Song et al. 2011). At low algal densities, it is relatively effective at eliminating the targeted algae with minimum damage to non-target algae (Tsai 2016). Copper sulfate can also be a problem in agriculture. It was found that 99% of the copper sulfate that was applied to a pond as an algaecide was present in the top 3cm of soil where crops were growing after the field had been irrigated with water from the treated pond (Salam and El-Fadel 2008). This indicates that plant toxicity could be a limiting factor to copper sulfate application. Copper is acutely toxic to fish, particularly at low concentrations in soft water (Woody and O'Neal 2012). Alternatively, chelated copper is much less toxic to fish (Closson and Paul 2014) and does not significantly alter the degradation or bacterial decomposition rates of microcystin (a cyanobacterial

hepatotoxin) (Iwinski et al. 2017), but can be transferred to local sediments within 2 days of application (Willis and Bishop 2016).

Sodium-based algaecides include a variety of sodium compounds. Sodium borate is often used in pools because it inhibits algae growth, reduces corrosion, and serves as a buffer (Birch 2013). More recently developed algaecides contain sodium carbonate peroxyhydrate, but this substance has been found to cause brain damage and liver inflammation in largemouth bass (Sinha et al. 2020). In large-scale public water treatment plans of lakes, sodium alum (or aluminate) is the preferred choice across the United States. Sodium alum works by inhibiting the release of phosphorous from sediments, thereby keeping phosphorous concentrations under control (Smeltzer 1990). In addition, the treatment is relatively inexpensive and the improved conditions persist for much longer than the aforementioned plans; sodium aluminate treatments last for years, compared to months or weeks with the other treatments (Smeltzer 1990). Like the other treatment plans, however, using this compound does have drawbacks. Aluminum from these treatments has been found in significant amounts in liver and kidney tissues in Rainbow trout (Buergel and Soltero 1983), and in some cases it has led to a long-term decline in water quality (Connor and Martin 1989).

Lithium is a possibly safer alternative to eliminate unwanted algae species because it is not expected to bioaccumulate and it has low toxicity to humans and the environment (Aral and Vecchio-Sadus 2008). Currently, the demand for lithium is increasing, as it is an important component of the lithium-ion batteries that power cell phones, laptops, power tools, and electric vehicles (Wanger 2011). It is predicted that the demand for lithium resources will be greater than the current supply by 2025, which

could lead to increased lithium mining and, subsequently, pollution (Wanger 2011). In marine waters with increased lithium pollution (approximately 0.08 mM of lithium) and warmer temperatures, the sea snail *Tritia neritea* experienced a decrease in feeding effectiveness as they took a longer amount of time to find food (Rodriguez et al. 2021). Most lithium pollution in aquatic environments results from the disposal of lithium batteries (LiBs), and is found primarily in Asian countries such as China (Zeng et al. 2015). Environmentally-friendly recycling methods utilizing non-toxic organic solvents and conversion to useful compounds can negate the pollution that occurs from the hydrolysis of the lithium hexafluorophosphate that is inside of the batteries, but these methods are expensive and not well-regulated (Bankole et al. 2013). In order to reduce lithium pollution from lithium-ion batteries, stronger government policy is required at local, national, and international levels (Kang et al. 2013).

Excess lithium has been removed from aquatic ecosystems through the processes of phycoremediation (removal through algae) and phytoremediation (removal through plants). Lithium ions have been successfully removed from freshwater environments using phycoremediation via the green algae species *Oocystis solitaria* (El-Naggar et al. 2019). Phytoremediation is a natural method to remove various contaminants and restore original environmental conditions, particularly in wetlands (Zhang et al. 2010). Although few studies of lithium phytoremediation have been conducted, several species [most notably Indian hemp (*Apocynum pictum*), Black Mustard *(Brassica juncea*), and the Common Grapevine (*Vitis vinifera*)] have been found to exhibit high levels of lithium tolerance and absorption, and therefore are great candidates for being cultivated for the purpose of phytoremediation (Jiang et al. 2018; Elektorowicz and Keropian 2015; Alagic

et al. 2016). Sword-leaf dogbane (*Apocynum venetum*) has been found to exhibit high lithium tolerance during germination, particularly to lithium chloride (Jiang et al. 2018). Additionally, Maize (*Zea mays*) has been found to take up large amounts of lithium in hydroponic conditions (Antonkiewicz et al. 2017).

In addition to lithium chloride, some antibiotics and potassium analogs of both sodium and lithium algal treatment compounds may be candidates for control of freshwater cyanobacteria. Streptomycin has been used for the purification of algal cultures (Droop 1967), inhibits the growth and pigment production in cyanobacteria (Kumar 1964), and inhibits the growth of toxin-producing marine dinoflagellates, albeit in extremely high concentrations (Wang et al. 2003). Other antibiotics like Tetracycline can adversely affect green algae more so than cyanobacteria in higher concentrations (Taskan 2016). Potassium salts, however, have been found to inhibit the growth of cyanobacteria, even at relatively low concentrations (Parker et al. 1997).

This experiment tests the effectiveness of lithium chloride as a method of chemical control of *Lyngbya wollei.* Additionally, the potential effects of high lithium concentrations on both *Lyngbya wollei* and a freshwater green algal species are investigated as well, as lithium pollution is an ever-increasing problem in certain parts of the world as the demand for lithium increases.

Materials and Methods

Microcosm experiments containing *Lyngbya wollei* cultured with increasing concentrations of lithium chloride were conducted. Samples of *L. wollei* were collected from Grassy Pond Recreational Area in Lake Park, Georgia and cultured in 300 mL Wheaton® BOD bottles in the phycology lab at Valdosta State University in Valdosta, Georgia. Cultures contained 200 mL of freshwater from the collection site, increasing concentrations of lithium chloride (0, 5, 10, 100, 200, and 400 mg/L), and samples of *L. wollei*. Samples were collected during December 2020, April 2021, and June 2021 to investigate whether there was a difference in treatment due to seasonal variability. Five samples were prepared at each concentration from each seasonal collection. In order to investigate the effects of increasing lithium chloride concentrations on freshwater green algae, a sample of green algae from Grassy Pond Recreational Area was included in the December 2020 collection. The green algae collection contained several species, although the majority were of the genus *Oedegonium*. Additionally, the effects of 3 antibiotics and potassium chloride at the same molar concentration as lithium chloride on L. wollei were investigated using samples with a second collection in June 2021. The water temperature at the time of collection for the April 2021 and June 2021 collections were 24.3°C and 28.1°C respectively. The masses of samples used in each replicate for each seasonal collection are presented in Table 1. The 5 mg LiCl/L and 10 mg LiCl/L concentrations were attained by adding 1 mL and 2 mL, respectively, of a 1 g LiCl/L stock solution along with pond water from the collection site as the diluent to reach a total solution volume of 200 mL. The 100 mg LiCl, 200 mg LiCl, and 400 mg LiCl concentrations were prepared by adding 2 mL, 4 mL, and 8 mL, respectively, of a 10 g LiCl/L stock

solution along with pond water from the collection site as the diluent to reach a total solution volume of 200 mL.

Sample	Mass (g)
L. wollei December 2020 LiCl	1.07 +/-0.05
Green Algae December 2020 LiCl	1.08 +/-0.05
L. wollei April 2021 LiCl	1.08 +/-0.05
L. wollei June 2021 LiCl	1.09 +/-0.06
L. wollei June 2021 Antibiotics	1.09 +/-0.06

Table 1. The masses of cultured samples from each seasonal collection (+/- std dev).

In the second collection in June 2021, samples were treated with 3 antibiotics (Streptomycin, Tetracycline, and Isoniazid) and potassium chloride. For streptomycin we used two concentrations- 20 mg/L or 0.3 mg/L- since these are the EC50 concentrations for a green algal species (*Chlorella vulgaris*) and a cyanobacterial species (*Microcystis aeruginosa*) respectively; streptomycin affects photosynthesis-related gene transcription and blocks electron transport (Qian et al. 2012). We used a concentration of 8 mg/L for tetracycline-this is the minimum inhibitory concentration (MIC) for some members of the family Oscillatoriaceae (Jones et al. 1973). A concentration of 0.2 mg/L was used for Isoniazid- twice the MIC for some strains of *Mycobacterium tuberculosis* (Suo et al. 1988). We used a potassium chloride solution with a concentration of 0.0236 M- equivalent to the molarity of lithium ions at the lowest effect concentration.

Each treatment concentration was cultured as replicates in 5 Wheaton® BOD bottles with Tilt-off™ caps. The experiment was repeated over the course of 3 different seasons (December, April, and June). Cultures were grown in the artificial growth chamber at Valdosta State University in Valdosta, Georgia at 25°C under 2 Philips® 150 W LED bulbs with a brightness of 2175 lumens and a photon flux of 99.12 µmol/s. During treatments, cultures were randomly rearranged using an online random number order generator (https://www.random.org/sequences/) every 24 hours for 7 days to ensure even light distribution across treatments.

After 7 days, the photosynthetic yield, maximal fluorescence, and baseline fluorescence of each sample was measured with pulse amplitude modulated (PAM) fluorometry using the Walz® Mini-PAM™ fluorometer. To assess damage to filaments, light micrographs were generated from transects of random samples of filaments from each treatment at a magnification of 400x. The images were then analyzed using the ImageJ software from the National Institutes of Health (NIH) (https://imagej.nih.gov/ij/). Data collected from each imaged included the length of damaged filament, the length of healthy filament, and the number of filaments. The length of damage of the filaments in each treatment group was expressed as a proportion out of the total length measured for data analysis. Damage was characterized as containing any of the characteristic patterns of damage presented and described in Figure 1.

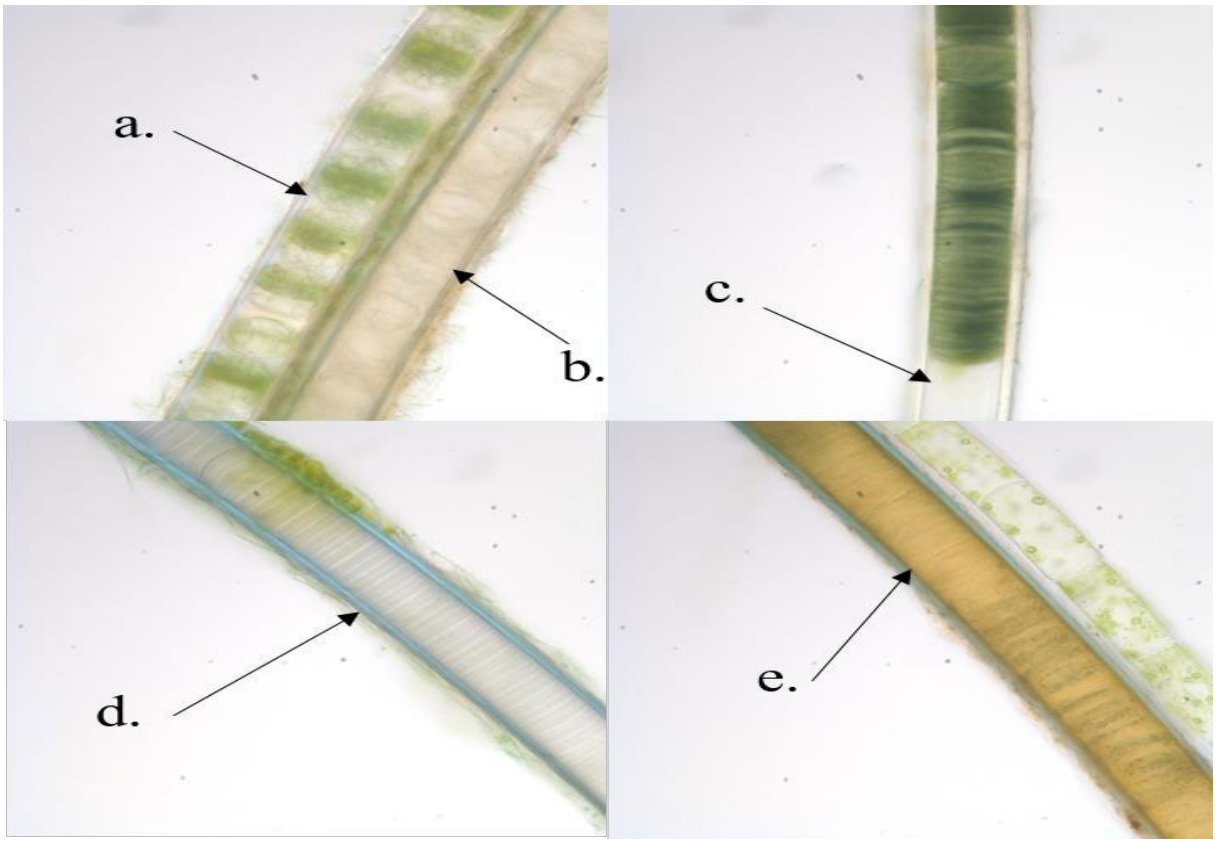

Figure 1. Characteristic patterns of damage, where the following are observed: a.) "banding" in which dead cells or empty sheaths are between green, healthy cells; b.) in which sheaths contain dead and disorganized cells; c.) in which empty sheaths are present; d.) in which sheaths contain dead cells in their original position, and e.) in which discoloration occurs and the cells may or may not be in their original position.

After the length of damage of each filament in each treatment sample was measured, one-way ANOVA was performed on the damaged proportions in each concentration group. Two-way ANOVA was also performed on the damaged proportions of the samples of *L. wollei* from the December 2020, April 2021, and June 2021 collection groups to determine if there was a difference among treatments due to seasonal variation. One-way ANOVA and two-way ANOVA were also performed on the photosynthetic yield and baseline fluorescence results from the PAM-fluorometer. The Analysis Toolpak™ from Microsoft® Excel was used to perform both one-way and two-

way ANOVA. In cases where a significant p-value was calculated, a post hoc Tukey's test was performed to determine which treatments significantly differed from the control. Various qualitative observations on the state of the treated samples were made and are presented in the results section.

Results

The one-way ANOVA results for the proportions of filaments damaged per treatment group of samples of each of the seasonal collections are summarized in Table 2.

Sample	Mean (proportion damaged)	P-value
L. wollei December 2020; LiCl	0.3 +/-0.29	<0.001
Green Algae December 2020 LiCl	0.18 +/-0.13	0.12
L. wollei April 2021; LiCl	0.32 +/-0.28	<0.001
L. wollei June 2021; LiCl	0.3 +/-0.3	<0.001

Table 2. The mean proportion of filaments damaged of samples from each seasonal collection. The mean is reported as (+/- std dev.).

In some treatments, the proportion of damaged filaments was less than that of the control group. In the samples from the December 2020 collection, the 10 mg LiCl/L treatment group had a mean proportion damaged (0.09 +/-0.03) that was healthier than that of the control (0.11 +/-0.04). This was also observed in the 5 mg LiCl/L treatment in the samples from the April 2021 collection (0.05 +/-0.003) as well as the 5 mg LiCl/L (0.08 +/-0.01), 10 mg LiCl/L (0.05 +/-0.002), and 100 mg LiCl/L (0.11 +/-0.01) treatments on the samples in the first June 2021 collection where the control means were (0.16 +/-0.03) and (0.2 +/-0.06) respectively, although none of these were significantly different from each other. In the samples of the December 2020 collection where the green algae was treated, the 5 mg LiCl/L treatment was the only one that had a higher mean proportion of filaments damaged (0.31 +/-0.02) than the control (0.24 +/-0.02). The

other treatments had a combined mean length of damage per filament of (0.13 +/-0.1). In the samples from the June 2021 antibiotic treatment, the mean proportion damaged across all samples was 0.33 +/-0.26 with a p-value of <0.001 and only the 8 mg Tetracycline/L treatment had a mean proportion of filaments damaged (0.14 +/-0.02) less than that of the control group (0.2 +/-0.06). Results from two-way ANOVA indicate no significant differences between samples due to seasonal variation ($p= 0.79$) or interactions between concentration and seasonal variation ($p= 0.72$).

The mean proportion of filaments damaged from samples of each treatment group of each seasonal collection is presented in Figure 2.

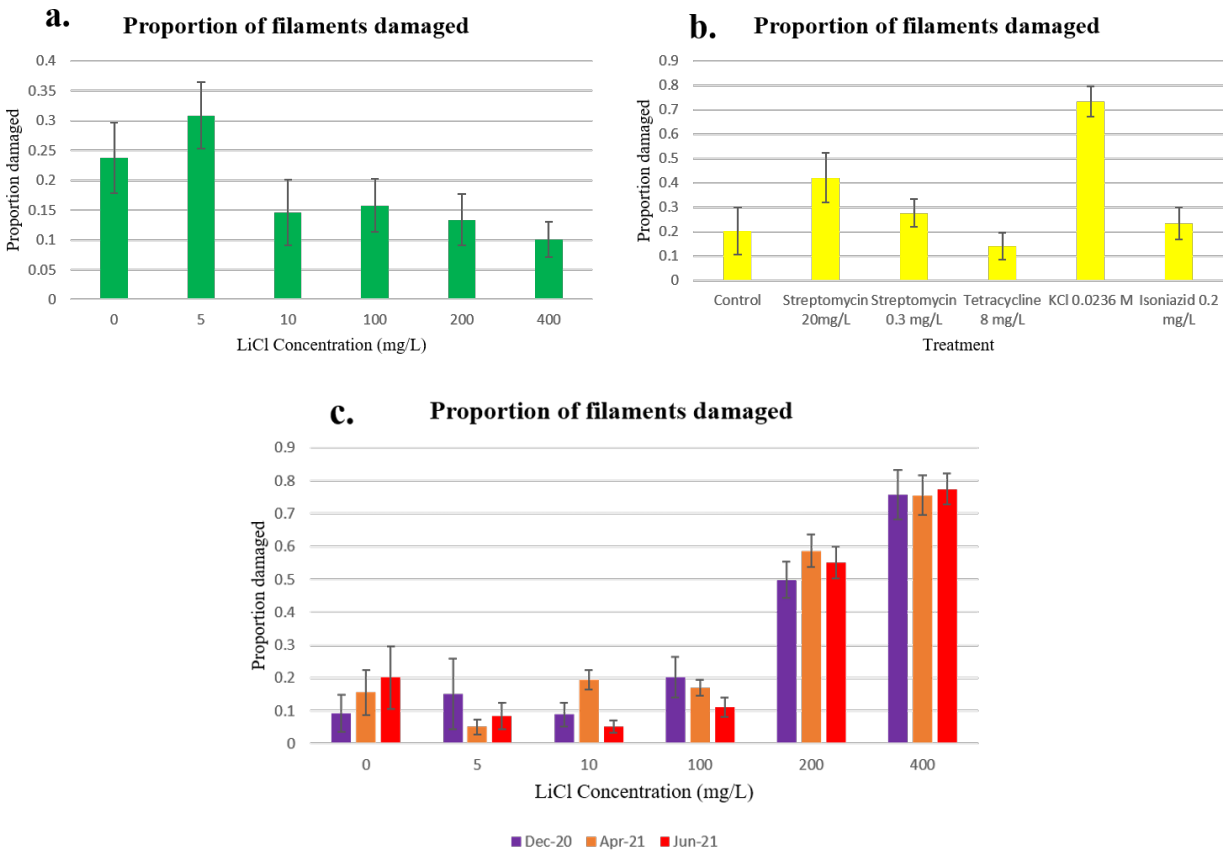

Figure 2. The proportion of filaments damaged in each treatment group of samples from each seasonal collection in which a.) represents the samples from the December 2020 green algae collection, b.) represents samples from the second June 2021 *L. wollei* collection, and c.) represents the samples from the December 2020, April 2021, and June 2021 *L. wollei* collections. For each microscopic analysis, n=5 and the error bars represent the standard error of the mean.

Color differences among the control group and the greatest LiCl concentration treatment group were observed as well, and these differences are illustrated in Figure 3.

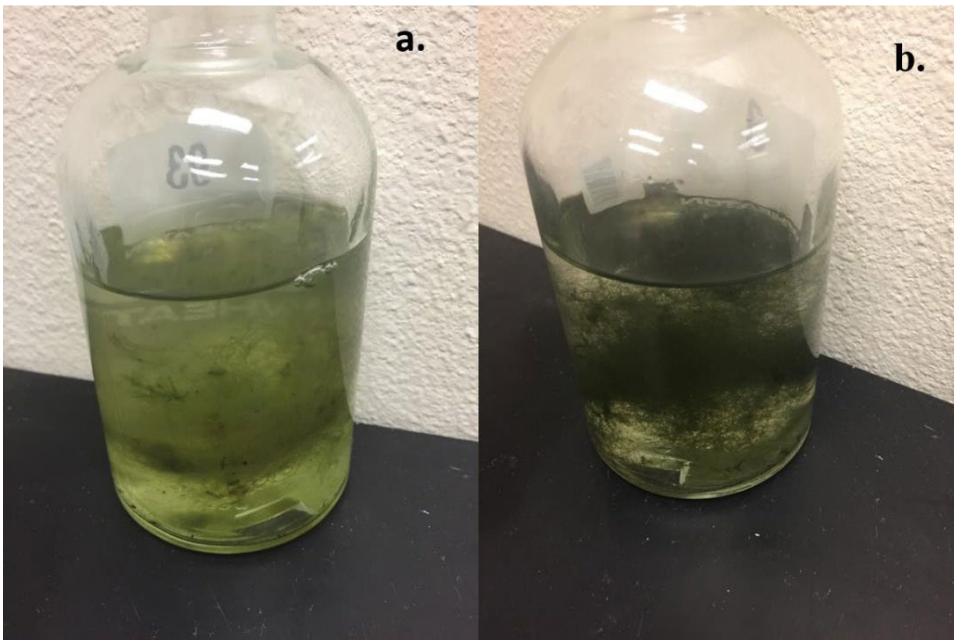

Figure 3. Color differences observed between the 400 mg LiCl/L treatment group (a.) and the control group (b.) in samples from the first June 2021 seasonal collection.

As seen in Figure 3, the treatment group receiving the highest concentration of LiCl (400 mg LiCl/L) exhibited a lighter shade of green than the control group, which exhibited a dark green color characteristic of healthy *L. wollei* filaments.

In order to determine which treatment groups were significantly different from the control group, Tukey tests were performed for samples of each seasonal collection and are summarized in Table 3. Because Tukey tests are pairwise comparisons and there were 5 treatment groups and a control group in each seasonal collection, only the treatment groups that were significantly different from the control are included in Table 3.

Sample	Control mean	Treatment	Treatment mean	Q-value
L. wollei December 2020	0.09 +/-0.13	200 mg LiCl/L	0.5 +/-0.12	7.58
L. wollei December 2020	0.09 +/-0.13	400 mg LiCl/L	0.76 +/-0.17	8.59
L. wollei April 2021	0.16 +/-0.15	200 mg LiCl/L	0.59 +/-0.11	8.33
L. wollei April 2021	0.16 +/-0.15	400 mg LiCl/L	0.76 +/-0.14	11.59
L. wollei June 2021	0.2 +/-0.21	200 mg LiCl/L	0.55 +/-0.11	5.91
L. wollei June 2021	0.2 +/-0.21	400 mg LiCl/L	0.77 +/-0.11	9.73
L. wollei June 2021 Antibiotics	0.2 +/-0.21	0.0236 M KCl	0.23 +/-0.15	6.32

Table 3. Tukey test results from the treatment groups that had a significantly greater proportion of filaments damaged than that of the control group mean. Q-crit value= 4.37.

In each seasonal collection, the 200 mg LiCl/L and 400 mg LiCl/L had a significantly greater proportion of filaments damaged than the untreated control groups. As evidenced by the results of the Tukey tests, the 400 mg LiCl/L treatment groups yielded greater mean length of damage per filament (0.76- 0.77) and, subsequently, greater q-values (8.59- 11.59) than the treatment groups receiving 200 mg LiCl/L (0.5- 0.59 and 5.91- 8.33, respectively). The treatment group receiving 0.0236 M KCl yielded a lower mean proportion of filaments damaged (0.23) compared to those of all lithium treatments and an intermediate q-value (6.32) in regards to those of both June 2021 lithium treatments. The greatest mean proportion of filaments damaged recorded of the 200 mg LiCl/L treatment group was in the samples from the April 2021 collection, while the greatest mean proportion of filaments damaged recorded for the 400 mg LiCl/L treatment group was from the samples in the June 2021 collection. The treatment group receiving the 0.0236 M KCl solution was only tested on the samples from the June 2021

seasonal collection. Also, it is noteworthy that there were no significant differences in mean proportion of filaments damaged between the 200 mg LiCl/L and 400 mg LiCl/L treatment groups regardless of which seasonal collection they originated from.

In an effort to determine if the treatments were affecting the photosynthesis of the samples, photosynthetic yield and baseline fluorescence was measured using PAM-fluorometry and analyzed with one-way ANOVA to test for differences among treatment groups and two-way ANOVA to test for differences in the samples due to seasonal variation. The ANOVA results of the PAM-fluorometry measurements of photosynthetic yield are summarized in Table 4, and the ANOVA results of the PAM-fluorometry measurements of baseline fluorescence are summarized in Table 5.

Sample	Mean	P-value
L. wollei April 2021 LiCl	242.4 +/-24.4	0.04
L. wollei June 2021 LiCl	324.5 +/-21.4	0.23
L. wollei June 2021 Antibiotics	305.7 +/-61.4	0.02

Table 4. The one-way ANOVA results of the phtotosyntheyic yield measured by PAM-fluorometry of samples from each seasonal collection. Means are reported as (+/- std dev.). No units are included, as photosynthetic yield and baseline fluorescence recorded as a result of PAM-fluorometry includes arbitrary units.

As presented in Table 4, the samples from the April 2021 and the second June 2021 seasonal collections contained treatment groups with statistically significant p-values. The treatment groups that were statistically significant from each other, however, did not include the control group in either seasonal collection in regards to photosynthetic yield. In the samples from the April 2021 collection, a Tukey test determined that the

treatment group receiving 100 mg LiCl/L (269.2) was significantly different from that of the treatment group receiving 400 mg LiCl/L (207.6; q-value=4.80; q-crit=4.37). In the samples from the second June 2021 seasonal collection that were treated with antibiotics, a Tukey test determined that the treatment groups receiving a solution of 0.0236 M KCl (375.4; q-value=4.56) and the treatment group receiving 0.2 mg Isoniazid/L (368.6; q-value=4.37) were significantly different from that of the treatment group receiving 0.3 mg Streptomycin/L (205).

Sample	Mean	P-value
L. wollei April 2021 LiCl	1190.4 +/-21.4	0.97
L. wollei June 2021 LiCl	1326.4 +/-93.7	0.17
L. wollei June 2021 Antibiotics	1316.6 +/-382.1	<0.001

Table 5. The one-way ANOVA results of the baseline fluorescence measured by PAM-fluorometry of samples from each seasonal collection. Means are reported as (+/- std dev.). No units are included, as photosynthetic yield and baseline fluorescence recorded as a result of PAM-fluorometry includes arbitrary units.

In the samples from the second June 2021 collection that were treated with antibiotics and potassium chloride, two treatment groups were significantly different from the control group in regards to baseline fluorescence: the 20 mg Streptomycin/L treatment group (2058.6 +/-242.9; q= 9.32) and the 8 mg Tetracycline/L treatment group (847 +/-112.6; q= 4.85;q-crit= 4.37).

Two-way ANOVA conducted on measurements of photosynthetic yield (Y) resulted in a significant p-value ($p < 0.001$), indicating a significant difference among samples due to seasonal variation. A Tukey test was performed to identify which

concentrations differed significantly from April to June 2021. The results are summarized in Table 6.

Concentration	April 2021 (Y)	June 2021(Y)	Q-value
5 mg LiCl/L	215.6 +/-34.94	313 +/-47.16	5.46
10 mg LiCl/L	235.4 +/-20.35	329.4 +/-40.07	5.27
200 mg LiCl/L	263.8 +/-30.72	370 +/-33.56	5.96
400 mg LiCl/L	207.6 +/-32.1	313.6 +/-37.58	5.95

Table 6. Tukey test results from measuring effects of seasonal variation on photosynthetic yield. Means are reported as (+/- std dev.). No units are included, as photosynthetic yield and baseline fluorescence recorded as a result of PAM-fluorometry includes arbitrary units. Q-crit=4.37.

Two-way ANOVA conducted on measurements of baseline fluorescence resulted in a significant p-value (p= 0.001), indicating a significant difference among samples due to seasonal variation. From April 2021 to June 2021, the treatment group of 100 mg LiCl/L (1202.4 +/-63.13- 1461.4 +/-76;q= 5.57; q-crit=4.37) contained the only baseline fluorescence measurements that were significantly greater. Results also indicate no significant differences due to interactions between concentration and seasonal variation for neither photosynthetic yield (p= 0.25) nor baseline fluorescence (p= 0.35).

Additionally, a few structural and ecological differences were observed in various treatment groups across seasonal collections. These differences are presented in Figure 4 and Figure 5, respectively.

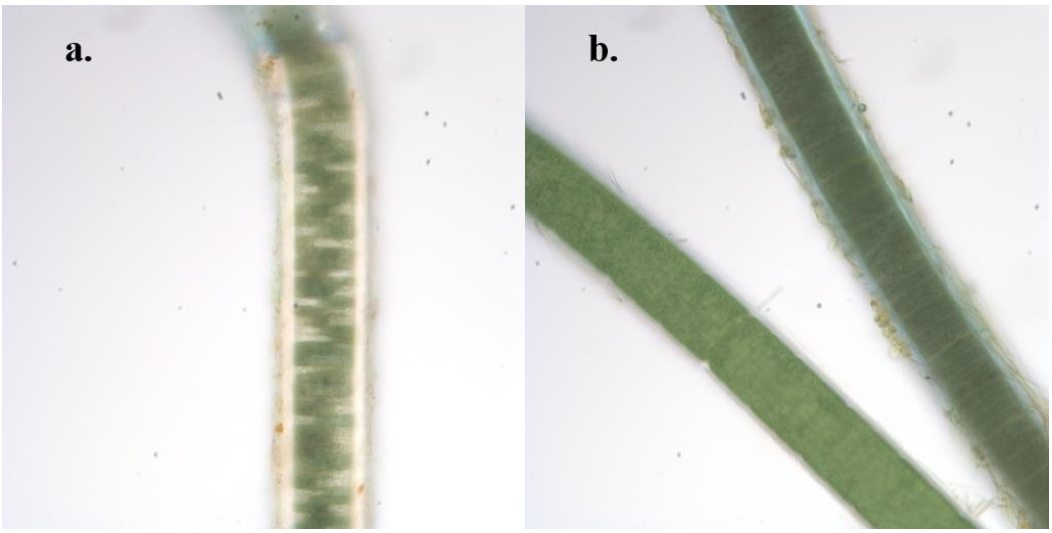

Figure 4. Structural differences observed in two LiCl treatment groups and the Isoniazid treatment group where a.) illustrates a "checkerboard" pattern on a *L. wollei* filament that was observed only 3 times throughout the course of the experiment, and b.) illustrates two healthy *L. wollei* filaments for comparison.

As seen in Figure 4, a "checkerboard" pattern was observed in 3 *L. wollei* filaments during the course of experimentation. Each "checkerboard" was observed twice in the December 2020 seasonal collection (once in the 100 mg LiCl/L treatment group and once in the 400 mg LiCl/L treatment group) and once in the second June 2021 seasonal collection (in the 0.2 mg Isoniazid/L treatment group). Since this pattern is difficult to characterize as damaged or healthy, the 3 observations of this pattern were omitted from data analysis. In the 8 mg Tetracycline/L treatment in the second June 2021 seasonal collection, each sample presented with a jelly-like feel which made the removal of excess water from the samples for PAM-fluorometry difficult. This characteristic is likely the result of tetracycline's mode of action.

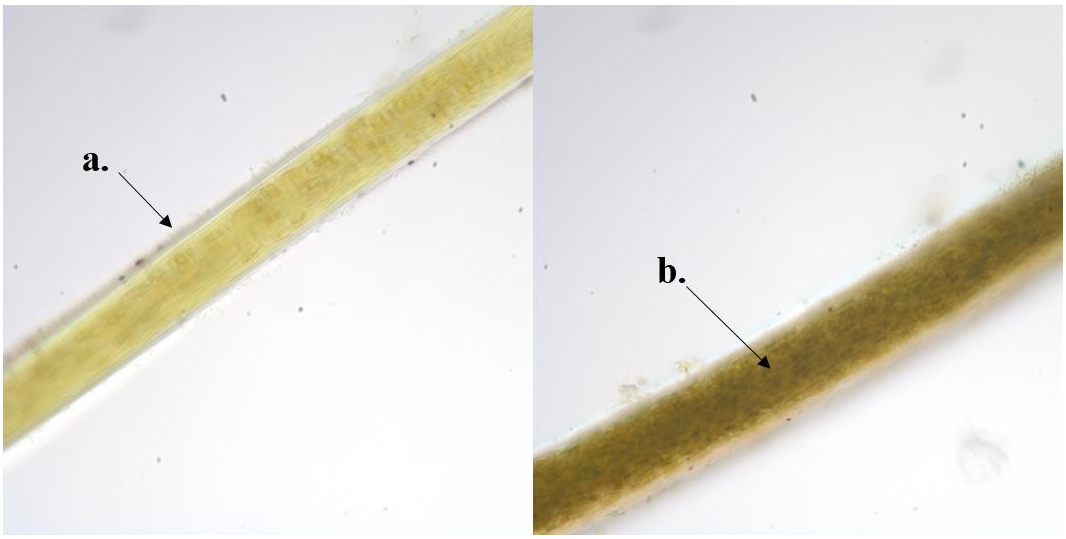

Figure 5. Opportunistic habitation of empty *L. wollei* fillaments by freshwater microorganisms. Here, a.) illustrates the mucilaginous sheath of the *L. wollei* filament and b.) illustrates freshwater microorganisms inhabiting the empty filament.

As seen in Figure 5, freshwater microorganisms were observed to inhabit empty filaments that were dead due to treatment with LiCl. This opportunistic habitation was observed in the treatment groups receiving 200 mg LiCl/L and 400 mg LiCl/L from the December 2020 seasonal collection after 14 days of treatment.

Discussion

The results of this experiment suggest that lithium chloride is a candidate for chemical control of filamentous cyanobacteria, particularly of the problematic species *Lyngbya wollei* that plagues the Southeastern United States. Additionally, seasonal variation in *L. wollei* does not influence the efficacy of lithium chloride's ability to damage cells. It should be noted, however, that the results of this experiment were obtained from microcosm cultures grown in a laboratory environment. In order to investigate the broad-scale ecological effects of lithium chloride treatment on a variety of species, large-scale outdoor mesocosm experiments would be ideal. Mostly, this is due to the fact that numerous freshwater species experience lithium toxicity and highly variable concentrations.

Lithium has long been used as treatment for mania and depression in patients with bipolar disorder (Sproule 2002). It was first used as a treatment for bipolar disorder by the Australian psychiatrist John Cade in 1949, as he speculated that the illness originated as a result of lithium deficiency in the body (Cade 1949). For a brief time in the 1940's, lithium chloride was even recommended as a substitute for table salt, but that came to end after several people died from lithium poisoning after using too much of it on their food (El-Mallakh and Jefferson 1999).

Small aquatic organisms such as planktonic crustaceans (e.g. *Daphnia magna*) and insect larvae experience toxic effects (primarily in the form of immobilization and embryo formation) from lower lithium concentrations (approximately 0.4- 1.7 mg/L) while many fish (e.g. *Morone saxatalis* (striped bass), *Gila elegans* (bonytail), and *Xyrauchen texanus* (razorback sucker)) do not experience toxic effects from lithium until

high concentrations of lithium (approximately 65- 156 mg/L) are achieved (Shahzad et al. 2017). Lithium is known to disturb the development of amphibian embryos in the cleavage and gastrulation stages (Birch 2012). In plants, lithium chloride is known to inhibit the nyctinastic closure of folioles in *Cassia fasciculata* (Partridge pea) (Gaillochet 1981) and inhibit the activity of myo-inositol-1-phosphatase in *Catharanthus roseus* (Madagascar periwinkle) (Nishida et al. 1993).

Interestingly, microscopic organisms seem to exhibit varying degrees of susceptibility of lithium chloride toxicity. At an incredibly high concentration of 1,000 mg/L, the growth rate of the single-celled green alga *Chlorella vannielli* was only reduced by 48% (Karlander and Krauss 1972). In bacteria, however, it is known to inhibit the growth of *Listeria* spp. (Cox et al. 1990), *Clavibacter michaganense* (Smidt and Vidaver 1986), and cause impairment in the rigidity of the cell envelope in *Escherichia coli* (Pitzurra and Szybalski 1959). In the unicellular ciliate *Tetrahymena thermophila*, lithium chloride affects the early stages of oral development (Jerka-Dziadosz and Frankel 1995).

In the United States, it is rare to find a body of water with a lithium concentration greater than 100µg/L (Sreekumaran et al. 1968), but closed lakes in western Mongolia have had concentrations recorded up to 100 mg/L (Shvartsev et al. 2012). Closed water systems like this with a greater amount of evaporation than rainfall are other great candidates for large scale ecotoxicological experimentation, as high lithium concentrations have already been demonstrated in these environments. It is difficult, however, to assess the specific impact that increased lithium chloride concentrations could have on aquatic biota, as the effects have only been studied on a few species.

The results of this study also indicate that the lowest effect concentration of lithium chloride for chemical control of *L. wollei* is somewhere between 100-200 mg LiCl/L. The European Chemical Agency (ECHA) reports that the EC50 of lithium chloride for the freshwater green algae *Desmodesmus subscipatus* is >400 mg LiCl/L (https://echa.europa.eu/substance-information/-/substanceinfo/100.028.375), which is consistent with the findings in this study, as the freshwater green algae tested in the December 2020 collection was a mixture of green algae genera also in Class Chlorophyceae, with the most prominent being *Oedegonium*. It is important to understand that a concentration of 200 mg LiCl/L had a negative effect (often resulting in death) on the *L. wollei* filaments. This concentration is the greatest concentration that can be efficiently absorbed and removed (98.71%) remediated from an aquatic environment with the freshwater green algal species *Oocystis solitaria* through the process of phycoremediation (El-Naggar et al. 2019). Some plants are able to withstand high lithium concentrations (up to 118 mg/L for corn (*Zea mays*) and >150mM for the Ethiopian mustard plant (*Brassica carinata*), respectively) (Li et al. 2009; Franzaring et al. 2016). Several plant species are able to accumulate lithium, but one of the most notable is the dogbane (*Apocynum pictum*) as it can accumulate >1,800 mg kg^{-1} in its leaves (Jiang et al. 2018).

In the context of the aforementioned ecotoxicological information and the two forms of bioremediation discussed, a two-step plan to chemically eradicate blooms of filamentous cyanobacteria (at least of the species *L. wollei*) can be proposed. The first step involves treating the aquatic system with a concentration of 200 mg LiCl/L. Assuming there is a small pond that is 10 m in width, 10 m in length, and 1 m in depth

(giving a volume of 100 m^3 or 100,000 L), a mass of 20 kg of LiCl would be required to reach that concentration. In comparison, the recommendation for copper sulfate application is much higher at 1.99 g/L (Raman and Cook 1988). In the same pond as previously described, a mass of 199 kg of copper sulfate would be required for treatment. Copper sulfate can be purchased for as little as $0.06/g in small quantities (Home Science Tools), and as little as $4.41/kg (Seed World) in bulk while lithium chloride can be purchased for as little as $0.020/g in small quantities (Carolina Biological) and as little as $294/kg in bulk (The Lab Depot). In the same example used above, the cost of treatment would be approximately $588 for lithium chloride and approximately $877 for copper sulfate. Thus, treatment with lithium chloride is most likely to only be cost-effective when treating small bodies of water. The prices provided for lithium chloride are those of laboratory-grade compounds. Copper sulfate is commonly used in algal treatments, so it is widely manufactured and sold in bulk as fine grade for lower prices. Additionally, lithium chloride treatment is recommended to be applied in the fall or winter, as many fish and amphibians breed and lay eggs in the spring and summer and the embryos and juveniles are the most sensitive to lithium toxicity.

The second step involves one or both of the bioremediation plans mentioned previously. After a body of water has been treated with 200 mg/L of lithium chloride, filamentous cyanobacteria cell death should be observed within 7 days. After 14 days, empty sheaths begin to become inhabited by other microorganisms. Since a concentration of 200 mg LiCl/L is not ideal, remediation must be started. Green algae such as *Oocystis solitaria* may be introduced into the aquatic environment to absorb and remove lithium

ions. Lithium-absorbing or lithium-tolerant plants such as corn, Ethiopian mustard, Black mustard, and the common grapevine may be planted along the shoreline.

In mammals, lithium is an essential nutrient and it has been used in the treatment of bipolar disorder (Jakobsson et al. 2017). Unlike other ions (like calcium, sodium, and potassium), lithium ions are not closely regulated in the human body, most likely due to the lack of lithium-specific binding sites and selectivity filters (Jakobsson et al. 2017). In swine, lithium chloride has been demonstrated to inhibit infections of *Mycoplasma hypopneumoniae* by preventing apoptotic cell death and the production of nitric oxide and superoxide anions, and reduced caspase-3 activity (Ishag et al. 2016). Presently, it is known that lithium reduces excitatory neurotransmission (dopamine and glutamate) and increases inhibitory (GABA) neurotransmission, but the specific mechanisms of its mood stabilization are still not well understood (Malhi et al. 2013). Lithium dosing is dependent on underlying medical conditions such as body weight, age, pregnancy, severity of episodes, and sex (Findling et al. 2010). On average, males are prescribed higher doses of lithium (approximately 900 mg/day in many clinical studies), older patients (age 55 and older) receive lower doses due to an increased sensitivity, and pregnant women who are on a lithium prescription do not experience increased or reduced doses regardless of the stage of pregnancy (Medhi et al. 2008); (Rej et al. 2014); (Wesseloo et al. 2017). In humans, lithium doses of 12-60g (171-857 mg/kg/day) can lead to coma, severe respiratory and cardiac complications, and death (Gosselin et al. 1984). Since the lethal dose of lithium for humans is quite high (at least 12g), it is not expected to be an issue for humans in bodies of water that are used recreationally, as it would be very difficult to achieve a concentration that would allow at least 12g of lithium to be ingested. Returning

to the example used previously, one would have to drink 60 L of water treated with 200 mg LiCl/L to ingest the lethal dose of lithium, which is very unlikely to happen.

The specific mechanism by which lithium chloride kills the cells of the filaments remains unknown. As seen in Figure 3, the cultures treated with the highest concentration of lithium chloride exhibited a lighter green color than the healthy control cultures. This would seem to indicate that lithium chloride may be affecting photosynthesis, but there were no significant differences in neither photosynthetic yield nor baseline fluorescence between the lithium-treated cultures and the control when analyzed with PAM-fluorometry. This is further supported by the results obtained from the green algal cultures. Interestingly, the treatment group receiving 100 mg LiCl/L was the only group that had a significantly greater baseline fluorescence from April to June, while it was the only group that did not have a significantly higher photosynthetic yield from April to June, indicating that the effect of seasonal variation on the filaments was greater than the effect of lithium concentration on the photosynthetic structures and processes. This increase in baseline fluorescence from April to June is likely due to the fact that environmental conditions characteristic of the summer months, such as sunlight and warmer temperatures, promote the growth of cyanobacteria such as *L. wollei* (Carmichael 2008; Speziale et al. 1991). Further conclusions about lithium's effect on chlorophyll a and the electron transport chain may not be drawn from the results presented here, as photosynthetic yield and baseline fluorescence were only recorded from the samples in April 2021 and June 2021.Comparisons between lithium ions and other ions that are major components of biomolecules may hold the key to understanding the mode of action of lithium. Lithium and magnesium have almost identical ionic radii and readily

substitute for each other in geologic formations, but this is surprisingly not observed in biomolecules such as chlorophyll a (Jakobsson et al. 2017). A possible action of lithium on G proteins has been suggested (Birch 2012), so it is possible that lithium may be interfering with a cyanobacterial signaling protein such as P_{II} that controls ammonium, nitrate, and urea uptake in cyanobacteria (Watzer et al. 2019) or overloading the Mrp system that includes a monovalent cation/antiporter system (Hagemann 2011).

Ecologically, a treatment plan using lithium chloride may result in dramatic changes in the dynamics of the aquatic system. As seen in Figure 5, the empty sheaths that are a result of the cells inside the filament being killed serve as habitat for freshwater microorganisms that are not very sensitive to lithium chloride. Therefore, it is possible that a lithium chloride treatment could not only remove cyanobacteria, but it could also provide a habitat for other freshwater microorganisms (that may or may not have been in competition with the cyanobacteria) to grow in the environment. Exactly which species are most likely and best suited to use these empty filaments is unknown, but it is important to note that this was only observed in the winter seasonal collection, and the species composition may be subject to temporal variability. Again, a large scale outdoor mesocosm experiment would be a great method to further explore which species would take advantage of this unique habitat. This phenomenon of "opportunistic habitation" has not been found to be documented in any published papers at the time of this writing. With respect to lithium pollution, the results are likely to be the same. In freshwater areas with increased lithium concentrations (200-400 mg/L), there are likely to be fewer cyanobacterial organisms and more green algae or other microorganisms. In areas with lithium concentrations >400 mg/L, assumptions cannot be made based off of the results

presented here. Additionally, the same premise cannot be extrapolated to include marine ecosystems due to a lack of experimentation.

As an alternative chemical control method, 3 antibiotics (Streptomycin, Tetracycline, and Isoniazid) were also tested on *L. wollei* samples. None of these samples, however, resulted in mean damage lengths per filament that were significantly different from that of the control. Streptomycin is a commonly prescribed antibiotic that inhibits protein synthesis in bacteria. Since *L. wollei* is a cyanobacterium and contains chlorophyll a, the EC50 concentration for both a green algal species (20 mg/L) and a colonial cyanobacterial species (0.3 mg/L) were tested. Although neither concentration resulted in statistically significant results in regards to the proportion of filaments damaged, the treatment group receiving 20 mg Streptomycin/L yielded a q-value that was the closest (2.61), indicating that the EC50 concentration of Streptomycin is >20 mg/L for *L. wollei*. The tetracycline treatment also did not yield statistically significant results, indicating that the EC50 concentration of tetracycline for *L. wollei* is >8 mg/L. A concentration of 30 mg tetracycline/L was found to slightly reduce protein quantity in the green algal species *Micractinium pusillum* (Bashir and Cho 2016), so the EC50 concentration of tetracycline is most likely between 8-30 mg/L or >30 mg/L. The cultures treated with tetracycline did, however, present with a jelly-like texture, which is most likely a result of tetracycline's mode of action that inhibits translation (Chopra and Roberts 2001). Isoniazid inhibits mycolic acid (long-chain fatty acids found in the cell walls of some bacteria) synthesis (Timmins and Deretic 2006). While treatment with this antibiotic at the concentration of 0.2 mg/L did not yield statistically significant results, a "checkerboard" pattern in the cells of one *L. wollei* filament was observed, which

appeared to be consistent with the early stages of damage. While literature reviews provide no evidence that cyanobacteria contain mycolic acid in their cells walls, concentrations similar to those recommended for other antibiotics above may be tested in order to determine if another mode of action is exerted upon cyanobacteria. In the comparisons of baseline fluorescence, the treatment group receiving 20 mg Streptomycin/L exhibited a greater baseline fluorescence than the control while the treatment receiving 8 mg Tetracycline/L exhibited a lower baseline fluorescence than the control, likely due to its bacteriostatic properties (Jones and Morrison 1962). Although the antibiotics were only tested on samples in one seasonal collection, they are a possible, but not promising, class of compounds for future experimentation. Wide-scale presence of antibiotics in bodies of fresh water can have negative effects, as it has led to antibiotic resistance (Kim et al. 2015) and likely originates from local wastewater sources (Czekalski et al. 2014).

The cultures receiving a 0.0236 M solution of potassium chloride yielded a significant difference that falls in the range of the lithium chloride treatments receiving a concentration of 200 mg/L. Potassium chloride has been demonstrated to inhibit growth of *Microcystis aeruginosa* at the concentration of 1 M (Parker et al. 1997), but the results of this experiment suggest its efficacy at a concentration much lower. Other salts such as sodium chloride are effective at inhibiting the growth of cyanobacteria by lowering the contents of superoxide dismutase, catalase (Zhang et al. 2013), and chlorophyll a (Deniz et al. 2011), but are probably most effective in small bodies of fresh water with high N:P ratios (Seale et al. 1987). Potassium analogs of both sodium and copper compounds used for algal control in freshwater ecosystems are promising avenues for future research.

Between concentrations of 1.5-6 mM, potassium sulfate has been shown to inhibit *Microcystis aeuriginosa* growth in pond water without any negative side effects to native fish (El Gammal 2008). Potassium tetraborate and potassium alum are excellent candidates for chemical control of cyanobacteria. They are soluble in water, but there is a dearth of information on their ecotoxicity available. Other lithium compound may be great candidates as well. Lithium sulfate, lithium tetraborate, and lithium aluminate have not been well-studied in regards to their chemical treatment of cyanobacteria potential, and these compounds are soluble, moderately soluble, and insoluble in water, respectively, which introduces another obstacle altogether.

Conclusion

Lithium chloride is an excellent candidate as a chemical control mechanism for blooms of the harmful cyanobacterial species *Lyngbya wollei*, particularly at the effect concentration of 200 mg/L. Although many aquatic organisms are susceptible to various concentrations of lithium toxicity, the affinity of lithium ions for absorption and removal via phycoremediation and phytoremediation make it a seemingly safe alternative to current chemical control mechanisms pending further large-scale testing. Seasonal variation among *L. wollei* had no effect on the efficacy of the effect concentration, but application would be recommended during the fall or winter in the Southeastern United States to minimize toxic effects to fish and amphibians, and the opportunistic habitation of empty filaments of *L. wollei* by freshwater microorganisms may provide insight into how the ecology of the system may change post-treatment. The mechanism by which lithium chloride damages and kills cells in the filaments of *L. wollei* is undetermined, but does not appear to be related to photosynthesis and should be further investigated. Antibiotics do not appear to be a reasonable chemical control method for freshwater cyanobacterial blooms, but potassium and other lithium analogs of current treatment compounds exhibit terrific potential.

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